Cytotoxic properties of clofibrate and other peroxisome proliferators: relevance to cancer progression

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CYTOTOXIC PROPERTIES OF CLOFIBRATE AND OTHER PEROXISOME PROLIFERATORS: RELEVANCE TO CANCER PROGRESSION

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Abstract

The biological activity of peroxisome proliferators (PPs) is mediated by a class of receptors, known as PPARs (PP-Activated Receptor), belonging to the nuclear receptor superfamily. Upon ligand binding, PPARs dimerize with retinoid receptors, translocate to the nucleus, recognize specific PP-responsive elements on DNA and transactivate a number of genes. Several processes are regulated by PPARs, such as mitochondrial and peroxisomal fatty acid uptake and β-oxidation, inflammation, intracellular lipid trafficking, cell proliferation and death. In addition, PPARs have been proposed to act as tumor suppressors or as tumor promoters, depending on the circumstances. In particular, PPs have been extensively studied for their hepatocarcinogenic action in rodents, most often ascribed to their antiapoptotic action. Recent evidence, however, has been provided about the antiproliferative, proapoptotic, and differentiation-promoting activities displayed by PPAR ligands. The present review will focus on the cytotoxic effects exerted by several PPs, among which clofibrate, on different types of tumor cells, with particular reference to the mechanisms of cell death and to their relevance to cancer induction and progression

Key words: peroxisome proliferators, PPAR, clofibrate, apoptosis, cancer

Introduction

Peroxisome proliferators (PPs) are structurally unrelated chemicals known for their ability to increase peroxisome number and size. The biological effects of PPs are mediated by the PP-activated receptors (PPARs), that belong to the nuclear hormone receptor superfamily. Three molecular forms of PPARs are known, namely PPARα, β/δ, and γ, differentially expressed in adult and embryonic tissues [1].

Among PPs, particular attention was given to fibrate derivatives, which include agents that have been and still are largely used as hypolipidemic drugs in view of their ability to lower plasma triglyceride levels by accelerating mitochondrial fatty acid β-oxidation through PPARα activation [2]. As well as other PPs, fibrate derivatives, among which clofibrate, were largely studied as hepatocarcinogens in rodents, also with reference to their antiapoptotic action. In this regard, however, several studies reported that clofibrate treatment induces massive apoptotic death in hepatoma cells [3, 4]. In different cell lines similar observation were also reported for other PPs such as nafenopin, perfluorooctanoic acid, and BR931 [3, 5, 6]. PP cytotoxicity is not restricted to cells of hepatic origin, since similar effects were observed in breast or lung cancer cell lines [7, 8] as well as in human keratinocytes, and normal or neoplastic T lymphocytes [9, 10; Penna et al., unpublished data]. In spite of these reports, however, the precise mechanisms whereby PP trigger apoptosis still remain to be elucidated.

PPs

Peroxisomes are single-membrane intracellular organelles, particularly abundant in the liver, but also detectable in other organs, including lung, muscle, nervous system, and kidney [11]. They contain several enzymes (catalase, oxidases, dehydrogenases), and are involved in essential cell functions such as respiration, gluconeogenesis, thermogenesis, and lipid metabolism. There are significant differences among species and tissues with regard to the levels of peroxisomal enzymes, the highest activities being reported for rat liver [11]. Peroxisome number and size can

be increased (from about 2% up to 25% of hepatocyte volume; [12]) by exposure to several compounds, including natural molecules (prostaglandins and long-chain fatty acids), synthetic compounds, among which fibrates, thiazolidinediones, and non-steroidal anti-inflammatory drugs, pesticides (diclofop-methyl, haloxyfop, lactofen, oxidiazon, 2,4,5-T), solvents (perchloroethylene, trichloroethylene), phthalate ester plasticizers [di(2-ethylhexyl)adipate (DEHA), di(2-ethylhexyl)phthalate (DEHP), butyl benzyl phthalate (BBP), or 2-ethylhexanol (2-EH)]. The structure of most PPs is characterized by a carboxyl group that can be already present in the native form or derive from its metabolism; however, also molecules without such a group can act as potent PPs. In this regard, these compounds could be divided into two groups: (i) molecules that become activated by forming either CoA or sulphate derivatives, and (ii) molecules that do not need activation to exert their bioactivity [reviewed in 13]. The effectiveness of these compounds in causing peroxisome proliferation may vary considerably. In addition, PPs cause a huge number of extraperoxisomal effects, such as hepatomegaly, altered activity of mitochondrial, microsomal, and cytosolic enzymes, as well as modulation of both hormonal and intracellular iron homeostasis [reviewed in 13, and 14].

PP effectiveness shows marked species differences, rodents being the most sensitive, while guinea pigs, monkeys, and humans appear relatively insensitive or nonresponsive at all [reviewed in 15]. Despite this low sensitivity, however, since humans are constantly exposed to PPs, because of therapeutic regimens, or in force of the contact with plasticizers, solvents, etc., the risk assessment becomes very important [16]. Epidemiological studies do not show peroxisome proliferation in patients treated with hypolipidemic fibrates [17], although contrasting evidence has also been provided [18, 19]. In general, most of the studies reported in the literature show the occurrence of beneficial, but not adverse, effects of PPs in humans.

Despite their structural diversity (Figure 1), PPs share the ability to induce pleiotropic effects that are generally reversible upon their withdrawal, provided that the exposure is not too prolonged (see below). The main target of these compounds is the liver, where they induce hypertrophy and hyperplasia, peroxisome proliferation, and enhanced activity of several classes of

enzymes, especially those associated with peroxisome and lipid metabolism [20, 21]. Among the enzymes induced by PP treatment, those involved in the peroxisomal β-oxidation show the most prominent increase, at both transcriptional and activation level [22].

While most of PP effects are mediated through the above cited three PPARs (α, β/δ, and γ), the observation that PP administration may also exert side effects such as angina crisis and increased serum levels of aminotransferase, creatine phosphokinase, or creatinine, suggest that PPs may also act by extra-receptor pathways. As an example, fibrates have been shown to freely enter the erythrocyte membrane and to bind hemoglobin, reducing its affinity for oxygen [23]. Other studies demonstrate that fibrates can disrupt mitochondrial respiration at the level of NADH cytochrome c reductase [13, 24]. Fibrates share the effect on the mitochondrial electron respiratory chain with a class of synthetic PPARγ ligands, the thiazolidinediones. However, fibrates specifically inhibit NADH cytochrome c reductase activity, leading cells to a metabolic shift towards anaerobic glycolysis and β-oxidation, resulting in significant hypotriglyceridemic and slight hypoglycemic effects. By contrast, thiazolidinediones, that are potent complex I inhibitors, reduce β-oxidation through the inhibition of NADH dehydrogenase activity, resulting in hypoglycemia associated with weak or absent hypolipidemic effect [24].

PPARs

PPARα was firstly discovered by Isseman and Green [25]. Soon after, two additional isoforms (β/δ and γ) were identified [reviewed in 12]. As for PPARγ, three variants have been described, namely PPARγ1, 2 and 3. PPARs are encoded by separate genes and exhibit different patterns of tissue distribution: PPARα is found in liver, kidney, heart, pancreas, skeletal muscle, brown adipose tissue, PPARγ mainly in brown and white adipocytes, while PPARβ/δ is ubiquitously expressed. They are involved in regulating energy expenditure, carbohydrate and lipid metabolism, tissue remodeling, inflammation, cell differentiation and proliferation. The physiologic ligands of PPARs were initially unknown, and the function of PPARα, in particular, was studied by means of

synthetic ligands, ranging from trichloroacetic acid to fibrates (clofibrate, bezafibrate, gemfibrozil) and plasticizers such as phtalates [25].

As for their structure, PPARs are similar to other nuclear receptors. Indeed, they contain a ligand binding domain, two transactivation domains (AF-1 and AF-2, in the N-terminal and in the DNA-binding regions, respectively), and a DNA-binding domain that interacts with the PP-response elements (PPREs). Upon ligand binding, a conformational modification in the transactivation domain allows dimerization with the obligate partner retinoid receptor (RXR) and improves the activation kinetics by favoring the release and recruitment of co-repressor and co-activator molecules, respectively. PPAR co-activators include the steroid receptor coactivator, PPARγ coactivator-1 (PGC-1), and CREB (cAMP-Response Element binding protein-Binding protein), while the Nuclear Receptor Corepressor (N-CoR) and Silencing Mediator for Retinoid and Thyroid hormone Receptors (SMRT) act as co-repressors (26, 27). The complex formed by the heterodimer PPAR/RXR and one of the co-activators interacts with DNA in correspondence of the PPREs, direct repeats of the hexanucleotide AGGTCA (but also AGGNCA or AGGTCA-3'), known as Direct Repeat (DR)-1 response element, separated by a single nucleotide [2] PPREs have been found in genes encoding molecules that regulate lipid metabolism and energy homeostasis as well as in genes coding for proteins involved in pathways relevant to cell survival, differentiation and proliferation [29].

Ligand-dependent PPAR activation may result not only in gene induction, but also in gene repression. As an example, DNA binding is inhibited by the interaction of co-repressors with PPARγ [30]. After ligand binding, the receptor dissociates from the co-repressor, leaving it available to bind and inhibit the STAT3 transcription factor [31]. In addition, PPARγ may directly mediate transrepression, through a mechanism that has been recently proposed to involve PPAR sumoylation, leading to stabilization of co-repressor recruitment [32]. By contrast, interaction of the co-repressor SMRT with PPARβ/δ does not result in interference with DNA binding [33]. In macrophages PPARβ/δ is involved in the regulation of MCP-1 expression by sequestering the transcriptional repressor B-cell lymphoma-6 (Bcl-6; [34]): PPARβ/δ ligand interaction results in Bcl-

6 release, which would inhibit MCP-1 transcription. Finally, PPARs have also been proposed to repress genes lacking PPREs. In this regard, PPARα has been shown to interact with the NF-κB p65 subunit, inhibiting gene transactivation [35], although not all NF-κB target genes are inhibited by PPARα activators. Generally speaking, PPAR-dependent transcriptional modulation is a dynamic process that involves the interaction of several proteins to form complexes in which both co-activators and co-repressors are rapidly exchanged [36]. In this regard, a finely tuned mechanism involving the cytosolic proteolytic system dependent on ubiquitin and proteasome ensures both assembly and turnover of these regulatory complexes. Although the three PPAR isotypes all are short-lived proteins degraded by the ubiquitin-proteasome system, their turnover is differently regulated. Upon ligand binding, PPARγ is ubiquitylated and degraded in a negative feedback loop, likely aimed at exerting a sort of autoregulation. By contrast PPARα ubiquitylation is prevented by ligand interaction, increasing receptor stability for a few hours, before a subsequent rapid proteasomal degradation [reviewed in 37]. Also PPARβ/δ levels are regulated by the ubiquitin-proteasome proteolytic system. Indeed, in the presence of proteasome inhibitors PPARβ/δ accumulates in the nucleus retaining transcription competence. In addition, PPARβ/δ half-life is significantly prolonged, in a reversible manner, by interaction with its ligands [reviewed in 37]. Such stabilization appears to rely on a selective block of PPARβ/δ ubiquitylation likely due to a conformational change caused by ligand binding that may inhibit the interaction with an ubiquitin ligase or favor the action of a deubiquitylating enzyme [37]. In addition to ligand-dependent receptor turnover, the ubiquitin-proteasome proteolytic system contributes to control PPAR activity in response to upstream signal transduction pathways. As an example, PPARγ phosphorylation by tyrosine or serine-threonine kinases enhances its proteasomal degradation [38]. Furthermore, PPARs appear to control the level of specific proteins by modulating the activity of the ubiquitin-proteasome system [reviewed in 37].

PPARs in physiology

PPARα is mainly involved in mediating the biological effects of both natural and synthetic PPs. In this regard, the hypotriglyceridemic effect exerted by fibrates appears to result from PPARα-mediated transcription of genes involved in lipid oxidative metabolism [39], and mice knock-out for PPARα are resistant to PP treatment in terms of hepatomegaly and peroxisome proliferation. PPARα induces lipoprotein lipase (LPL) expression, reduces the expression levels of apolipoprotein C-III (ApoC-III), a natural LPL inhibitor, stimulates the uptake of fatty acids and their conversion to acyl-CoA derivatives [40]. In addition, PPARα increases the expressions of ApoA-I and ApoA-II, raising HDL cholesterol levels in humans [41]. Among PPARα target genes, those involved in the peroxisomal β-oxidation were first identified [42]. Peroxisomal β-oxidation results from two distinct metabolic pathways, one inducible by PPs that utilizes straight-chain acyl-CoAs as substrates and one constitutive, mainly at least, that catalyzes the oxidation of branched fatty acyl-CoA esters. The genes coding for the enzymes involved in the first pathway (fatty acyl-CoA oxidase, enoyl-CoA hydratase/L-3-hydroxyacyl-CoA dehydrogenase, and 3-ketoacyl-CoA thiolase) are regulated at the transcriptional level by PPARα [42]. In addition, PPARα also regulates the transcription of the gene encoding the sterol carrier protein x, an enzyme endowed with thiolase activity, involved in the non-inducible peroxisomal β-oxidation pathway [43]. Other metabolic routes are under the control of PPARα, including mitochondrial β-oxidation, microsomal ω-oxidation, ketogenesis and lipoprotein metabolism [reviewed in 44]. PPARα activation has also been shown to reduce the inflammatory response, mainly through down-regulation of cyclooxygenase-2 expression/activity or decreased activation of NF-κB. Finally, activated PPARα also appears to improve cardiovascular risk factor and cardiovascular outcome [reviewed in 44].

PPARγ plays a pivotal role in adipocyte differentiation, modulates metabolism and inflammation, and displays marked antiproliferative effects. PPARγ activation by both natural or synthetic ligands regulates fat storage in the adipose tissue, results in improved insulin sensitivity and atherosclerosis, and in the control of cell proliferation, macrophage function, and immunity [45]. Similar to PPARα, PPARγ attenuates the inflammatory response by forming a complex with nuclear NF-κB p65 subunit, causing its export to the cytoplasm and reducing the expression of

proinflammatory genes [46]. PPARγ can be activated by various dietary ligands such as polyunsaturated fatty acids, eicosanoids, glutamine, spicy food or flavonoids. In addition, also anti-inflammatory properties of probiotics may be mediated through this receptor [47]. PPARγ is the main target of the thiazolidinedione class of insulin-sensitising drugs, and besides these synthetic ligands, it is activated by naturally occurring compounds, such as prostaglandin J2 derivatives and polyunsaturated fatty acids [48]. When phosphorylated by extracellular signal-regulated kinases (ERK)-1/2 and c-Jun N-terminal kinase (JNK), PPARγ affinity for ligands is reduced [49]. PPARγ activation may be impaired also by binding of the ERK-activating kinase MEK to the AF-2 motif, leading to release from PPREs and nuclear exit [50]. In this regard, polyunsaturated fatty acids and their metabolites result in direct activation of PPARγ, but being able to activate the MEK/ERK pathway, they also contribute to events leading to PPARγ inhibition.

PPARβ/δ is a powerful regulator of fatty acid utilization and energy homeostasis in several tissues. Consistent with such a role, PPARβ/δ protein content is increased during physiological conditions characterised by elevated fatty acid utilization, such as physical exercise or fasting. Recent evidence indicates that PPARβ/δ can promote lipid accumulation by increasing the expression of genes involved in lipid uptake, and by repressing genes involved in lipid metabolism, inflammation, atherosclerosis, obesity and cancer [51]. PPARβ/δ is characterised by a large size ligand-binding pocket, which allows interaction with a greater variety of activators when compared with other nuclear hormone receptors. PPARβ/δ is activated by polyunsaturated fatty acids, prostacyclin and synthetic molecules, such as phenoxyacetic acid derivatives (e.g. GW 501516 and GW 0742; [48]), and has been proposed to act as a lipid sensor, being activated by fatty acids derived from very-low-density lipoprotein [52]. The PPARβ/δ specific agonist GW501516 reduces hypertriglyceridemia and raises high-density lipoprotein levels in obese subjects. In addition, both weight gain and insulin resistance induced in mice by assumption of a high-fat diet can be reduced by administration of GW501516 [53]. Finally, as reported for the other PPARs, also PPARβ/δ agonists are endowed with anti-inflammatory properties, leading to inhibition of LPS-inducible genes in murine peritoneal macrophages [54].

PPs and PPARs in carcinogenesis

In 1976, Reddy and coworkers described for the first time the development of hepatic tumors in mice treated with nafenopin. The subsequent observation that liver carcinogenesis could be obtained also by using other PPs, led to include these compounds among the non-genotoxic carcinogens [21], admittedly able to promote cancer development in force of their mitogenic effect, without causing direct DNA damage. The role of PPs and their receptors, PPARα in particular, in rodent hepatocarcinogenesis has been clearly defined by experiments showing that treatment of mice with Wy-14,643 increased hepatocyte proliferation and development of liver tumors, while PPARα-null mice were refractory to these effects [55].

Several mechanisms have been proposed to explain PP-induced carcinogenesis in rodents. The increase in peroxisome volume and number caused by PPs results in high H_2O_2 levels [56], likely due to the enhanced expression/activity of peroxisomal enzymes [42]. The role of oxidative stress in PP carcinogenicity is confirmed by the observation that ciprofibrate-induced hepatocarcinogenesis is inhibited by antioxidant treatments [57].

Increased hepatocyte proliferation in response to PPARα activation likely contributes to PP-induced carcinogenesis. In this regard, chronic administration of nafenopin to mice has been shown to significantly increase liver weight, and hepatic DNA synthesis, eventually resulting in the development of hepatocellular carcinomas [20]. Similarly, liver hyperplasia and increased levels of mRNAs encoding proteins involved in cell cycle regulation, such as cyclin-dependent kinase (CDK) 1 and 4, cyclins B1 and D1, c-myc and proliferating cell nuclear antigen (PCNA), were observed in mice treated with Wy-14,643 or bezafibrate [55, 58]. Finally, inhibition of apoptosis may also play a role in PP-induced hepatocarcinogenesis. The importance of apoptosis in the process of carcinogenesis has been well established since the functional characterization of the *Bcl-2* oncogene, and defective apoptosis is now commonly accepted to contribute to cancer development not less than deregulated cell production. In this regard, the tightly controlled

homeostatic mechanisms that link cell proliferation and apoptosis in normal liver are disrupted during hepatocarcinogenesis. Apoptosis is a rare event in the normal rodent liver [59], but it is rapidly activated during the regression of liver hyperplasia elicited by different stimuli [60] and can be experimentally induced by the administration of agonist anti-Fas antibodies *in vivo* [61]. Low or undetectable apoptotic rates occur in preneoplastic or early neoplastic liver lesion of animals treated with different tumor promoters, such as PPs. Consistently, nafenopin has been shown to promote cell survival in hepatocyte cell cultures [62, 63]. PPARα activation due to different PPs results in inhibition of liver apoptosis, especially in the presence of TNFα [64].

Despite the observations that PPs act as hepatocarcinogens in rodents, epidemiological studies suggest that they are unlikely to exert carcinogenic effects in humans [15]. Indeed, long term exposure of dyslipidemic patients to fibrates, used as hypolipidemic drugs, does not reveal any increase in the incidence of hepatocellular carcinomas [65]. Such difference in the susceptibility to carcinogenesis may rely on the lower PPARα expression in human liver with respect to mouse (less than one tenth; [15, 66]). This could increase the availability of PPREs for other members of the nuclear receptor superfamily, resulting in down-regulation of both peroxisome proliferation and the related consequences. This consideration apart, several PPARα variants have been detected in human cells that can act as dominant negative regulators of receptor activity [15, 67]. The expression of one of these PPARα variants prevents the suppression of apoptosis exerted by nafenopin in FAO hepatoma cells [67]. In addition to PPARα variants, also sequence differences in PPREs have been discovered, introducing another possible mechanism to explain the different sensitivity to PP carcinogenic effect between humans and rodents. Indeed, the occurrence of differences on PPREs may well interfere with the affinity of PPAR binding to DNA, resulting in inefficient gene transcription [68]. An interesting approach to investigate the mechanisms underlying the different response to PPs among species has been developed by generating mice genetically deficient for PPARα, while able to specifically express the human PPARα under the control of tetracycline [69]. These mice exposed to Wy-14,643 or fenofibrate show a response comparable to wild-type animals as for the effects on lipid metabolism. By

contrast, no changes could be observed in the expression of genes involved in cell cycle regulation or in the development of hepatocarcinomas [70]. Recently, Wy-14,643 has been shown to inhibit, by an unknown mechanism, the expression of mice hepatic microRNA let-7C, which appears to act as a tumor suppressor. Indeed, let-7C degrades c-*myc* mRNA by binding to 3'-UTR of the c-*myc* gene: thus, the reduced expression of let-7C induced by Wy-14,643 results in increased levels of c-myc as well as of the oncogenic mir-17 cluster [71]. Comparable results have been obtained on a different model based on PPARα knock-out mice expressing the complete human PPARα gene on a P1 phage artificial chromosome genomic clone [72].

A number of studies showed that PPARγ is expressed in different types of tumor cells, and that PPARγ ligand-dependent activation results in inhibition of cell proliferation, promotion of differentiation and induction of apoptosis [44 and refs. therein]. In addition, similarly to PPARα, PPARγ down-regulates the activity of a variety of transcription factors. Evidence has been provided that PPARγ is involved in the regulation of neoplastic growth, while in some types of cancer it has been proposed to act as a tumor suppressor. Indeed, PPARγ exerts antiproliferative effects on gastric, bladder, prostate, colon and breast cancer, and is involved in regulating cell proliferation in malignant melanoma [reviewed in 44]. PPARγ ligands down-regulate the expression of cyclooxygenase-2, an enzyme that has been involved in tumorigenicity and invasiveness in human colon cancer [73]. In this regard, thiazolidinediones such as troglitazone and rosiglitazone have been shown to inhibit cell proliferation and to induce cell cycle arrest of rat intestinal epithelial cells, mainly by reducing cyclin D1 expression [74]. Treatment of MCF7 cells with troglitazone also inhibits cell proliferation by decreasing the expression of several regulators of pRb phosphorylation, such as cyclin D1, CDK2, CDK4, and CDK6 [75]. In addition, treatment of HCT15 colon cancer cells with troglitazone induces the expression of p21Cip1/Waf1, a negative regulator of cell cycle progression [76]. Similar inhibition of cell proliferation has been reported for CaCo2 cells exposed to rosiglitazone [77]. PPARγ activation inhibits tumor growth also by blocking angiogenesis [78] and by inducing apoptosis in cancer cells [73]. Finally, administration of the PPARγ synthetic agonist GW7845 has been shown to prevent the development of chemically-

induced mammary tumors [79]. In contrast, however, a recent study reports that the development of mammary tumors is significantly enhanced in transgenic mice expressing a constitutively active PPARγ [80], while PPARγ activation by thiazolidinediones appears to increase tumor development in a genetic model of colon cancer [81]. In addition, PPARγ has been found to induce the production of hepatocyte growth factor, thus favoring tumor growth [82].

Also PPARβ/δ, similarly to PPARγ, has been reported to play opposite roles in carcinogenesis. Recent evidence supports the hypothesis that PPARβ/δ promotes tumor progression: HCT116 PPARβ/δ-null cell lines grow more slowly than wild-type cells, and exhibit a decreased ability to form tumors when inoculated into nude mice [83]. The promotion of tumorigenesis exerted by PPARβ/δ appears to rely on increased cell proliferation. Indeed, a few years ago this receptor has been proposed to be involved in the progression of colon cancer, being a potential target of the adenomatous polyposis coli (APC)/*β*-catenin pathway [84]. In this regard, exposure of mice carrying a mutated form of the APC gene to a selective agonist of PPARβ/δ (GW501516) causes a significant increase in polyp number in the small intestine [83]. This observation appears in contrast with another report showing that PPARβ/δ is not required for polyp formation in the same mouse model [85]. However, since the most prominent effect of GW501516 is on polyp size, PPARβ/δ activation is likely to affect primarily the rate of polyp growth rather than to initiate polyp formation. The same PPARβ/δ agonist accelerates mammary carcinogenesis induced chemically or by hormone stimulation in mouse [86].

PPARβ/δ is a downstream target gene of Ras/Raf/MAPK and extracellular signal-regulated kinase (ERK) kinase (MEK)/ERK pathways, both involved in the stimulation of cell proliferation [87]. Finally, PPARβ/δ also appears to inhibit apoptosis and promote keratinocyte survival. In this regard, in cells undergoing apoptotic death PPARβ/δ expression levels decrease [88].

On the other side, stimulation of differentiation has been reported in keratinocytes exposed to PPARβ/δ ligands [89], and consistently, PPARβ/δ deficient mice exhibit a marked hyperplastic response when treated with TPA [90].

PP cytotoxicity: relevance to cancer progression

Many PPs are known to modulate carcinogenesis by directly interfering with the rate of cell proliferation and death. Particularly intriguing in this regard is the observation that, depending on cell type and experimental conditions, PPs exert opposite effets on cell survival, inhibiting cell death or triggering apoptosis, a mechanism activated to get rid of excess, damaged, or infected cells, fundamental for the maintenance of tissue homeostasis. This process is controlled largely by pro-apoptotic and pro-survival factors generated by signal transduction pathways, starting with the initiation of death signals at the plasma membrane or intracellularly, and proceeding through complex cytoplasmic networks before reaching the end point [91, 92]. Apoptotic signaling mainly converges in the activation of intracellular proteinases known as caspases, which propagate death signals by cleavage of key substrates [93]. In mammalian cells, two well-characterized apoptotic pathways activate initiator caspases, which then cleave and activate effector caspases to execute cell death [94]. Death receptor-dependent apoptosis (extrinsic pathway) follows ligand binding to one of the six known death receptors (Fas, TNFR1, DR3, DR4/TRAIL-R1, DR5/TRAIL-R2, and DR6). This leads to recruitment of adaptor proteins [Fas-Associated Death Domain (FADD) and TNF Receptor-Associated Death Domain (TRADD)] and of procaspase-8. In force of autoproteolytic mechanisms, the latter becomes activated, eventually resulting in effector caspase activation and cell death [95]. On the other hand, stress pathways may culminate in the release to the cytosol of mitochondrial proteins such as cytochrome c and Smac-Diablo, leading to formation of the apoptosome complex and sequential activation of caspases 9 and 3. This mitochondrial dependent (intrinsic) pathway is negatively regulated by several antiapoptotic factors such as Bcl-2 and Bcl-x_L. Intrinsic and extrinsic apoptotic pathways can be strictly interconnected since the former can also be activated by death receptors through caspase-8 mediated cleavage of the pro-apoptotic factor Bid: cleaved Bid translocates to mitochondria where it facilitates cytochrome c release and caspase-9 activation [96].

Ligands specifically acting on the different PPAR moieties have been reported to activate apoptotic cell death (Table 1). In this regard, a conspicuous number of studies is focused on PPARγ activation [97], while just few have investigated the mechanisms of cytotoxicity exerted by PPARα ligands. The present review is an attempt to fill this gap providing a comprehensive report on the state of the art in PPARα ligand-induced apoptosis, with particular reference to clofibrate.

A number of studies indicate that treatment with clofibrate promptly induces massive and typical apoptosis in hepatoma cells of both murine (Yoshida AH-130) and human origin (HepG2; [3]). Such observations have been extended also to other PPs: nafenopin is able to induce apoptotic death in the AH-130 cells [3], while perfluorooctanoic acid and BR931 exert cytotoxic effects on the HepG2 hepatoma cell line [5, 6]. A recent study shows that in clofibrate-treated pigs the expression of the pro-apoptotic protein Bax is up-regulated, while the levels of the anti-apoptotic factor Bcl-x_L are reduced, which may provide the basis for an increase of apoptotic cell death. In agreement with this report, treatment of mice with Wy-14,643 has been shown to up-regulate the expression of pro-apoptotic genes and to down regulate that of anti-apoptotic factors in the liver, while PPARα-null mice appear protected against these modifications [98]. The same study shows that PPARα activation by Wy-14643 also increases liver sensitivity to apoptosis induced by treatment with agonist anti-Fas antibodies.

The apparent contradiction existing in terms of PP effects on apoptosis (inhibition or induction) probably depends on cell type, ligand type and concentration, duration of treatment. As an example, monoethylhexylphthalate at low or high doses, respectively, exerts anti- and proapoptotic effects on hepatocyte cultures [64]. On this line, data reported on cultured guinea-pig hepatocytes show suppression, no effect, or promotion of apoptosis [99-101]. Comparable results have been obtained on human hepatocytes [102, 103]. PP cytotoxicity is not restricted to cells of hepatic origin, since similar effects have been observed in breast or lung cancer cell lines [7, 8] as well as in human keratinocytes, lymphocytes, and monocyte-derived macrophages [9, 10, 104, 105].

Irrespective of the mechanisms leading to PP-induced cell death, the observation that clofibrate and other PPs exert a marked lethal action on cells of both rodent and human origin leads to the conclusion that these agents should be carefully reevaluated for their potential use as antineoplastic drugs. This suggestion is further supported by previous reports showing that PP action as tumor promoters is characterized by two apparently contradictory events. In this regard, reduced number and size of preneoplastic lesions have been reported after short term PP treatment, while, in the same models of chemical hepatocarcinogenesis, prolonged exposure results in the appearance of large and numerous tumors [106]. The protective effect exerted by PP short term treatment is likely due to a selective advantage of certain cell populations over others. Indeed, during hepatocellular carcinoma progression, preneoplastic lesions are characterized by extensive remodeling and marked cell turnover [106-108]. Stimulation with PPs may interfere with the balance of signals regulating cell survival and death, potentiating the latter, thus resulting in preneoplastic lesion regression.

Mechanisms of PPARα ligands cytotoxicity

The mechanisms involved in apoptosis induced by PPARα ligands, and by clofibrate in particular, are far from being elucidated. In cells of hepatic origin clofibrate is apoptogenic at 0.5-1 mM concentration, which is apparently quite high. However, similar or even higher concentrations have been used in biochemical studies on tissue cultures [109] and 200-300 mg/kg b.w. per day is the usual dose in protocols for chemical hepatocarcinogenesis in the rat [see 110]. In pharmacological terms, optimal therapeutic plasma levels of clofibrate have been reported to be around 0.5 mM [111, 112].

Many of the pleiotropic effects of clofibrate may be relevant to the induction of apoptosis. As an example, this type of cell death may be caused by inhibition of macromolecular synthesis: in this regard, PPs have been demonstrated to suppress both DNA and RNA synthesis [113], as well as to inhibit the activity of HMGCoA reductase, a key enzyme in the process of isoprenoid biosynthesis [114]. Consistently, previous results obtained in our laboratory show that the mRNA

level and enzymatic activity of HMGCoA reductase as well as the cholesterol content in mitochondria are reduced in Yoshida AH-130 hepatoma cells soon after clofibrate treatment, while cell death can be attenuated by supplementing cells with mevalonate, the reaction product of HMGCoA reductase [115]. In addition to cholesterol synthesis, inhibition of this enzyme affects the biosynthesis of isoprenoid units. In this regard, some studies show that inhibition of mevalonate synthesis exerted by lovastatin, a *bona fide* inhibitor of HMGCoA reductase, impairs Ras isoprenylation and activation of the MAP kinase cascade [116], or RhoA geranylgeranylation [117], resulting in down-regulation of cell proliferation. Consistently, lovastatin has been shown to suppress cell growth and to induce apoptosis in several tumor types [118]. No alterations of protein farnesylation could be observed in clofibrate-induced AH-130 apoptosis, since the localization of p21ras remains comparable between control and treated cells. In addition, clofibrate-induced apoptosis still occurs in the presence of a farnesyltransferase inhibitor. These results suggest that inhibition of protein farnesylation is not involved in clofibrate cytotoxicity, the more so because it requires a prolonged time to produce cell death [115]. Similar observations have been made on human keratinocytes exposed to clofibrate [9].

PPARα induction appears to play a crucial role in the modulation of apoptosis. In JM2 rat hepatoma cells, characterized by very low levels of PPARα, clofibrate treatment inhibits cell proliferation, without inducing apoptosis [119]. By contrast, in human HepG2 hepatoblastoma cells, clofibrate-induced apoptosis is associated with increased PPARα levels [115]. Another study reported that PPARα activation enhances hepatocyte apoptosis [98]. By contrast, in FAO hepatoma cells PPARα has been shown to be required for the protection against nafenopin-induced death [120]. Results obtained in our laboratory show that, at least in clofibrate-induced AH-130 cell death, PPARα does not seem to be involved, consistently with the rapidity of the apoptotic response [115]. Where occurring, PPARα expression is enhanced early, well before the decrease in HMGCoA reductase [9, 121, 122]. However, when keratinocytes are treated with MK886, an inhibitor of PPARα, clofibrate-induced apoptosis is not completely prevented, suggesting the involvement of other mechanisms [88].

One of the effects exerted by PPs is the increase in number and size of peroxisomes, associated with increased production of reactive oxygen species (see above). Cell death by oxidative stress may pertain to apoptosis or necrosis, depending on the extent of cell damage. As for apoptosis, reactive oxygen species play a critical role, directly, in force of their macromolecule damaging activity, but also indirectly. Results obtained in our laboratory show that lipid peroxidation is not modified in clofibrate-treated AH-130 cells with respect to controls [123]. Consistently, both the AH-130 or the Jurkat T cells cannot be rescued from clofibrate-induced death by antioxidants such as BHA, BHT or N-acetyl-cysteine [Penna et al., unpublished data].

Clofibrate-induced apoptosis is caspase-dependent. This has been reported by studies on L6 rat skeletal myoblasts and IM-9 human lymphoblasts, where 2 to 24h clofibrate exposure results in caspase activation [10, 124]. Caspases 3, 8, and 9 are very rapidly activated by clofibrate in AH-130 hepatoma cells, and pretreatment with different caspase inhibitors affords a significant protection against apoptosis. The extent of the protective effect is limited with inhibitors of caspases 8 or 9, extensive with the caspase-3 inhibitor DEVD-cho, and virtually complete with the poly-caspase inhibitor z-VAD-fmk [4]. Results quite similar, although with a markedly different kinetic of activation as for caspases 8 and 9, have been obtained on Jurkat cells [Penna et al., unpublished data].

The observation that caspase 9 is activated by clofibrate, at least in the AH-130 and in the Jurkat cell lines, suggests that the mitochondrial apoptotic pathway is activated in clofibrate-induced cell death. Indeed, soon after clofibrate exposure mitochondrial membrane potential rapidly falls down, and pro-apoptotic factors such as cytochrome c and, at least in the AH-130 cells, Smac-Diablo are released [4, 115, Penna et al., unpublished data). Despite these observations, however, a causal relation between the intrinsic pathway of apoptosis and clofibrate cytotoxicity could not be convincingly unproven. Indeed, caspase-9 inhibition or treatment with cyclosporin A, a drug that inhibit both mitochondria depolarization and cytochrome c release, only partially prevent apoptosis in the AH-130 cells, and are completely uneffective in the Jurkat cell line [4, Penna et al., unpublished data].

Although clofibrate-induced apoptosis involves a marked activation of caspase 3, the apical caspase(s) remain to be identified since caspases 8 and 9 are unlikely to play such a role. In this regard, clofibrate has been reported to inhibit the transport of newly synthesized proteins from endoplasmic reticulum (ER) to Golgi, causing the retrograde movement of Golgi constituents back to the ER [125]. These findings suggest that clofibrate may induce ER stress. In the last years ER stress has been proposed to trigger apoptosis through a mechanism depending on caspase 12 [126], involving both initiator (2, 8, 9) and effector (3, 4, 7) caspases [127]. ER-induced cell death appears associated with recruitment of TNF receptor-associated factor 2 (TRAF2) and JNK phosphorylation [128]. In addition, proteins of the Bcl2 family are involved in the regulation of apoptosis by ER stress [129]. Finally, ER stress is associated with inhibition of protein synthesis, and enhanced phosphorylation of eIF2α is a reliable marker of such condition [130]. An early and marked increase in eIF2α and JNK phosphorylation can be observed after clofibrate treatment in both AH-130 and Jurkat cells. These changes are not associated with procaspase 12 cleavage, while a marked activation of caspase 2 occurs. Although these findings may suggest a role of apical caspase for caspase 2, pre-treatment with an inhibitor selective for this caspase only partially rescues both cell lines from clofibrate-induced death [4, Penna et al., unpublished data], leaving the 'apical caspase matter' still unclear.

Both ER stress and mitochondrial depolarization induce Ca^{2+} mobilization that may result in activation of calpains, a class of Ca^{2+}-dependent proteases that in the last years, have been associated with procaspase 12 processing and activation [131]. More recently, clofibrate treatment of L6 murine skeletal myocytes has been reported to induce apoptotic death due to Ca^{2+}-dependent caspase 12 activation [124]. Calpain activation *in vivo* can be detected by estimating the cleavage of specific physiological substrates such as fodrin, the 130 kDa Ca^{2+}-ATPase, and calpastatin. Clofibrate treatment of both AH-130 or Jurkat cells results in extensive fodrin cleavage [4, Penna et al., unpublished data]. However, the involvement of calpains in clofibrate-induced apoptosis is questioned by the observation that pre-treatment of Jurkat cells with a specific calpain inhibitor does not afford any protection against cell death [Penna et al., unpublished data].

Conclusions

PPs and PPARs are crucially involved in the regulation of several metabolic pathways, such as lipogenesis and fatty acid oxidation. In addition, they play a role in cell processes including proliferation, differentiation and death. Particularly intriguing in this regard is their cytotoxic activity, that may be relevant to negatively interfere with tumor progression. The precise mechanisms underlying the cytotoxicity of PPs, PPARα ligands in particular, remain to be elucidated in part. Indeed, while the caspase-dependency of the process is clear, the molecular trigger leading to caspase activation remains to be determined. In this regard, activation of the intrinsic apoptotic pathway and occurrence of ER stress appear to play a potential role.

Induction of apoptosis is certainly one of the main reasons underlying the therapeutic potential of PPAR ligands in oncology. However, other PP bioactivitites may be equally relevant. In this regard, down-regulation of the inflammatory response, obtained by inhibition of both COX2 and NFκB activity, appear of particular interest. In addition, the observation that PPAR*α* exerts both antiangiogenic and antitumorigenic activities [reviewed in 132] suggests that this class of receptors could be considered as tumor suppressors.

In view of the above reported observations, PPs may be proposed as new antiblastic drugs, characterized by both low toxicity and cost. PPARα ligands might also be used in association with other drugs, such as COX2 inhibitors or statins [10, 133]. As for the latter, however, caution should be used in view of the potential muscle toxicity [134, 135]. Finally, PPAR-dependent signal transduction pathway may be effectively modulated in the future by means of siRNA or antisense methodologies, or by selective PPAR modulators, able to exert most of the benefits, while reducing the adverse effects displayed by full PPAR agonists [136].

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Table 1. Apoptosis induction by PPAR ligands

PPARα	
Clofibrate, clofibric acid	B and T-lymphocytes, keratinocytes, liver and ovarian cancer, T-cell leukemia [3, 9, 105, 137, Penna et al., unpublished)
Wy-14,643	hepatocytes [98]
perfluoroctanoic acid	liver cancer cells [5]
Nafenopin	liver cancer cells [3]
BR931	liver cancer cells [6]
conjugated linoleic acid	liver cancer cells [138]
TDZ18	lymphocytic leukemia [139]
MCC-555	colon cancer cells [140]
PPARβ/δ	
GW0742	colon cancer cells [141]
PPARγ	
TDZ (troglitazone, rosiglitazone, ciglitazone, pioglitazone, TDZ18, MCC-555)	osteoblasts, promyelocytic leukaemia, lymphocytic leukaemia, colon, breast, liver, thyroid, ovarian, and lung cancer [7, 8, 139, 140, 142-144]
15d-PGJ2	neurons, B-lymphocytes, B-lymphoma,promyelocytic leukaemia, colon, breast, thyroid and lung cancer (142, 143, 145-147]
DEHP	Sertoli cells [148]

DHEP = Di(2-ethylhexyl) phthalate; TDZ = thiazolidindione;

15d-PGJ2 = 15-Deoxy-Delta(12,14)-prostaglandin J2.

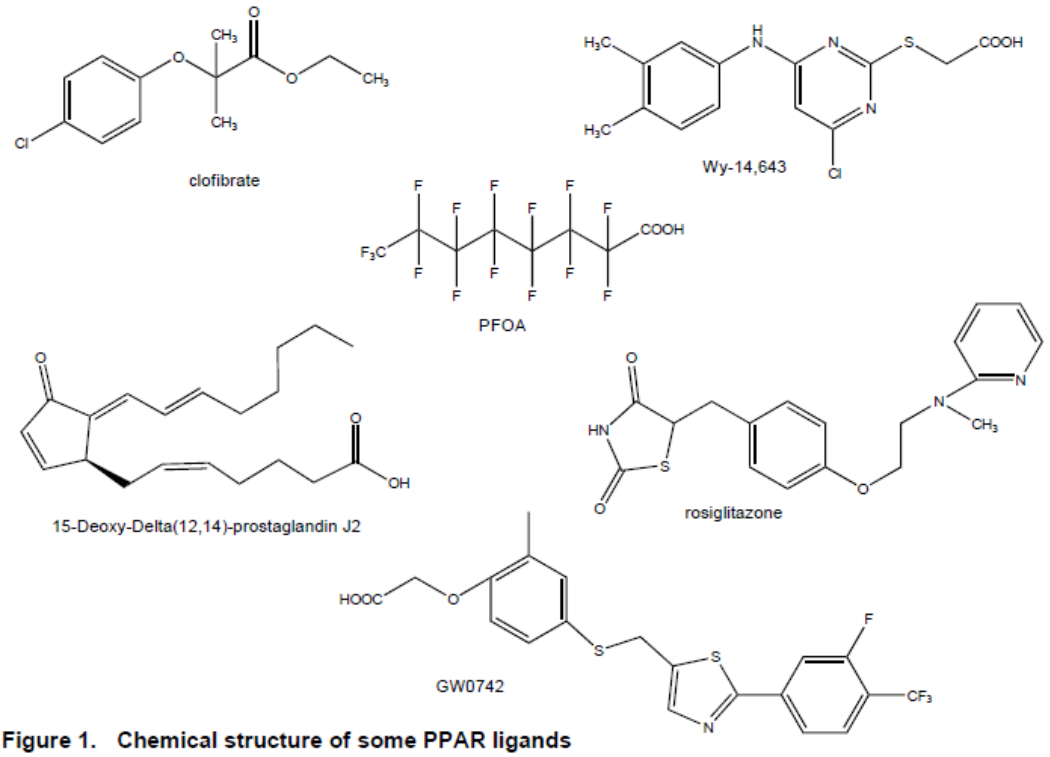

Figure 1. Chemical structure of some PPAR ligands

PFOA = perflororctanoic acid